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## **Stabilization of a prion strain of synthetic origin requires multiple serial passages**

Makarava, Natallia ; Kovacs, Gabor G ; Savtchenko, Regina ; Alexeeva, Irina ; Budka, Herbert ; Rohwer, Robert G ; Baskakov, Ilia V

**Abstract:** Transmission of prions to a new host is frequently accompanied by strain adaptation, a phenomenon that involves reduction of the incubation period, a change in neuropathological features and, sometimes, tissue tropism. Here we show that a strain of synthetic origin (SSLOW), although serially transmitted within the same species, displayed the key attributes of the strain adaptation process. At least four serial passages were required to stabilize the strain-specific SSLOW phenotype. The biological titration of SSLOW revealed a correlation between clinical signs and accumulation of PrP(Sc) in brains of animals inoculated with high doses (10<sup>-1</sup>-10<sup>-5</sup>) diluted brain material), but dissociation between the two processes at low dose inocula (10<sup>-6</sup>-10<sup>-8</sup>) diluted brain material). At low doses, several asymptomatic animals harbored large amounts of PrP(Sc) comparable with those seen in the brains of terminally ill animals, whereas one clinically ill animal had very little, if any, PrP(Sc). In summary, the current study illustrates that the phenomenon of prion strain adaptation is more common than generally thought and could be observed upon serial transmission without changing the host species. When PrP(Sc) is seeded by recombinant PrP structures different from that of authentic PrP(Sc), PrP(Sc) properties continued to evolve for as long as four serial passages.

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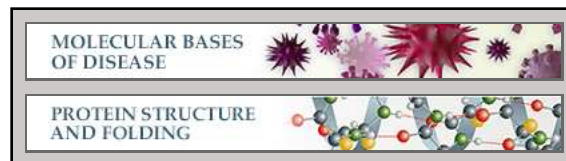
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**Molecular Bases of Disease:**  
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Stabilization of a prion strain of synthetic origin requires multiple serial passages

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Running title: Adaptation of synthetic prions

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Keywords: prions; prion diseases; prion titration; dose-response; hamster; Protein Misfolding Cyclic Amplification

**Background:** Strain adaptation accompanies cross-species transmission of prions.

**Results:** Adaptation of a strain of synthetic origin was observed within the same host species.

**Conclusion:** When induced by recombinant PrP fibrils, PrP<sup>Sc</sup> properties evolve over multiple serial passages within the same host.

**Significance:** The phenomenon of prion strain adaptation is more common than generally thought.

## SUMMARY

Transmission of prions to a new host is frequently accompanied by strain adaptation, a phenomenon that involves reduction of the incubation period, a change in neuropathological features and, sometimes, tissue tropism. Here we show that a strain of synthetic origin (SSLOW), while serially transmitted within the same species, displayed the key attributes of the strain adaptation process. At least four serial passages were required to stabilize the strain-specific SSLOW phenotype. The biological titration of SSLOW revealed a correlation between clinical signs and accumulation of PrP<sup>Sc</sup> in brains of animals inoculated with high doses ( $10^{-1}$ - $10^{-5}$  diluted brain material), but dissociation between the two processes at low dose inocula ( $10^{-6}$ - $10^{-8}$  diluted

brain material). At low doses, several asymptomatic animals harbored large amounts of PrP<sup>Sc</sup> comparable to those seen in the brains of terminally ill animals, whereas one clinically ill animal had very little, if any PrP<sup>Sc</sup>. In summary, the current study illustrates that the phenomenon of prion strain adaptation is more common than generally thought and could be observed upon serial transmission without changing the host species. When PrP<sup>Sc</sup> is seeded by recombinant PrP structures different from that of authentic PrP<sup>Sc</sup>, PrP<sup>Sc</sup> properties continued to evolve for as long as four serial passages.

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are a family of fatal infectious neurodegenerative diseases (1). The infectious agent of prion disease consists of a prion protein in its abnormal, self-replicating,  $\beta$ -sheet rich state (2). Assistance of cofactors including lipids and polyanions was shown to be essential for converting the normal, cellular isoform of the prion protein, PrP<sup>C</sup>, into the highly infectious, disease-related form, PrP<sup>Sc</sup> (3,4).

During several decades of extensive exploration of TSEs, our knowledge about the disease has been continuously shaped by the choice of laboratory TSE models. Among the most commonly used TSE models are several well-

characterized rodent-adapted TSE strains (5). All currently employed rodent strains were originally isolated from natural TSE sources including sheep, goat or mink and adapted to hamsters or mice, sometimes via passaging through intermediate species (6-12). Frequently, the original TSE isolates appeared to consist of strain mixtures (9,13-17). Nevertheless, multiple individual prion strains were isolated using the laborious procedure of cloning via limiting dilution and serial passaging within the same host (13,18). While several stable TSE strains with a broad spectrum of disease phenotypes were produced by adapting TSE isolates to rodents, the strains with short incubation times were preferred in the majority of subsequent studies as a matter of convenience.

In recent studies, an alternative approach for generating TSE in rodents emerged. This approach involves conversion of PrP<sup>C</sup> or recombinant PrP (rPrP) into PrP<sup>Sc</sup> in non-seeded Protein Misfolding Cyclic Amplification (PMCA) reactions (3,4,19). When hamster PrP<sup>C</sup> was used as a substrate, PMCA-derived strains displayed disease phenotypes that closely resembled those of the commonly used hamster-adapted 263K or Hyper strains of natural origin (19,20). We introduced a different experimental strategy that does not involve PMCA, but relies on treatment of rPrP with chemical denaturants for converting rPrP into amyloid fibrils (21-25). Using this approach a panel of prion strains (SSLOW, LOTSS, S05) was created in Syrian hamsters by inoculating fibrils produced *in vitro* from full-length rPrP (25-27). While recognized as a TSE infection, the disease produced by these strains of synthetic origin displayed phenotypes notably different from those of natural TSE strains or PMCA-derived strains. All synthetic strains that originated from rPrP fibrils were characterized by long incubation time to disease, slow progression of clinical disease and peculiar clinical and neuropathological features.

Because no bona fide PrP<sup>Sc</sup> was found in the preparations of rPrP fibrils, a new mechanism for the transmissible prion diseases referred to as deformed templating was proposed (26,27). According to this mechanism, rPrP fibrils while being structurally different from PrP<sup>Sc</sup>, nevertheless, can give a rise to bona fide PrP<sup>Sc</sup> via deformed templating. The unique disease phenotype expressed by the strains of synthetic

origin supported the hypothesis that these TSEs originated from rPrP fibrils with unique structures.

Considering the lessons learned from the cross-species adaptation of prion isolates of natural origin, here we asked the question of whether the unique disease phenotype displayed by the synthetic strains is stable during serial transmission or represents only an adaptation stage, after which a phenotype transforms into one of the phenotypes common for the TSEs of natural origin. Using SSLOW (Synthetic Strain Leading to OverWeight) (25), we found that the key SSLOW-specific characteristics including long incubation time to disease, slow progression of clinical disease, SSLOW-specific neurotropism and PrP<sup>Sc</sup> deposition in the form of large subependymal plaques were all maintained during serial transmission. Unexpectedly, in a manner similar to adaptation that accompanies cross-species prion transmission, multiple serial passages were required to stabilize the SSLOW-specific disease phenotype despite the fact that transmission was conducted within the same species. Furthermore, the biological titration revealed a correlation between clinical signs and accumulation of PrP<sup>Sc</sup> in brain at high dose inocula ( $10^{-1}$ - $10^{-5}$  diluted brain material), but no correlation between the two processes at low dose inocula ( $10^{-6}$ - $10^{-8}$  diluted brain material). At low doses, several animals showed large amounts of PrP<sup>Sc</sup> in the absence of clinical signs, whereas one clinically ill animal showed very small amounts of PrP<sup>Sc</sup> if any. These results support the hypothesis that two distinct processes, one being responsible for PrP<sup>Sc</sup> replication and another for accumulation of toxic species, exist (28). In summary, the current study illustrates that the phenomenon of prion strain adaptation is more common than previously thought and that it could be observed upon serial prion transmission without changing the host species.

## EXPERIMENTAL PROCEDURES

**Ethics statement** - This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore (Assurance Number A32000-01; Permit Number: 0312020).



**Bioassay** - For serial transmission, 10% BHs prepared by sonication in PBS, pH 7.4 (25), were dispersed by an additional 30 sec of sonication immediately before inoculation. Each Syrian hamster received 50  $\mu$ l of 10% BH inoculum intracerebrally under 2% O<sub>2</sub> / 4 MAC isoflurane anesthesia. Starting from the third month postinoculation, hamsters were observed daily for disease using a 'blind' scoring protocol. Animals were euthanized upon reaching terminal stage or, if no signs of clinical disease were observed, at 685 days post inoculation. Their brains were removed aseptically and saved for analysis and subsequent passage. End-point titration bioassay was performed as previously described (29).

**Proteinase K digestion** - Brains were collected aseptically and cut in half with disposable scalpels. One half was used to prepare 10% BHs in PBS, while the second half was stored at -80°C for future analysis or fixed in formalin for histopathology. Homogenization was performed on ice, in PBS, pH 7.4, using glass/Teflon tissue grinders attached to a cordless 12V compact drill (Ryobi). The brains were ground at low speed until homogeneous and stored at -80°C in 1 ml aliquots. For the Proteinase K digestion, 10% BH was diluted to 5% with the same volume of 4% sarcosyl in PBS, supplemented with 50 mM Tris, pH 7.5, and digested with 20  $\mu$ g/ml PK for 30 min at 37°C with 1000 rpm shaking using a Delfia plate shaker (Wallac) placed in a 37°C incubator. PK digestion was stopped by adding SDS sample buffer and heating the samples for 10 min in a boiling water bath. After loading onto NuPAGE 12% BisTris gels and transfer to PVDF membrane, PrP was detected with 3F4 (epitope 109-112) antibody.

**Protein misfolding cyclic amplification with beads** - 10% NBH from healthy hamsters was prepared as described before (26) and used as a substrate for PMCA with beads (PMCAb) (30). To detect PrP<sup>Sc</sup> in the brains of inoculated animals, 10  $\mu$ l of 10% BH were added to the 90  $\mu$ l of NBH. The standard sonication program consisted of 30 sec sonication pulses delivered at 50% power efficiency applied every 30 min during a 24 hour period. For each subsequent round, 10  $\mu$ l of the reaction from previous round were added to the 90  $\mu$ l of a fresh substrate. Each PMCAb reaction was carried out in the presence of three 3/32" Teflon beads (SmallParts #B000FMUEXG). To maintain the temperature during PMCAb, the microplate

horn together with its sound enclosure was placed in a 37°C incubator (VWR, model 1915). For biochemical titrations of PrP<sup>Sc</sup> in the brains of inoculated animals, an aliquot of 10% BH was sonicated within a 0.2 ml thin-wall PCR tube for 30 sec at 50% efficiency inside microplate horn of a Misonix-4000 sonicator before serial log dilution into conversion buffer. 10  $\mu$ l of each dilution were added to the 90  $\mu$ l of NBH, and PMCAb was carried out as described above. To analyze PMCAb products, 10  $\mu$ l of the sample were supplemented with 5  $\mu$ l SDS and 5  $\mu$ l PK, to a final concentration of SDS and PK of 0.25 % and 50  $\mu$ g/ml, respectively, followed by incubation at 37°C for 1 hour. The digestion was terminated by addition of SDS-sample buffer and heating the samples for 10 min in a boiling water bath. Samples were loaded onto NuPAGE 12% BisTris gels, transferred to PVDF membrane, and probed with 3F4 antibodies.

**Histopathological studies** - Tissues from SSLOW-inoculated hamsters were processed as described previously (25). Briefly, formalin fixed brain halves divided in the midline (from passage 2, 3, and 4) and spleen (from passage 2 and 4) were stained with hematoxylin-eosin, with monoclonal anti-PrP antibody 3F4 (1:1000, Covance, Berkeley, CA, USA) for detecting PrP, and with monoclonal anti-glial fibrillar acidic protein (GFAP; 1:3000, Dako, Glostrup, Denmark). Blocks were treated in formic acid (96%) prior to embedding in paraffin. For detection of disease-associated PrP, we applied a pretreatment of 30 minutes hydrated autoclaving at 121°C followed by 5 minutes in 96% formic acid. We evaluated all tissues for the presence of inflammation and PrP immunoreactivity, and the brain for the presence of spongiform change and degree of gliosis. Degree of spongiform change, neuronal loss and gliosis, and intensity of PrP immunostaining was semiquantitatively evaluated (0: none; 1: mild; 2: moderate; 3: severe). Lesion profiles were obtained by averaging scores of spongiform change, neuronal loss and gliosis for each anatomical region and animal group.

## RESULTS

*SSLOW maintains its strain-specific disease phenotype during serial transmission* - To test whether stabilization of SSLOW-specific strain features requires multiple passages and to establish a stable disease phenotype, serial transmission of

SSLOW using Syrian hamsters was conducted. The incubation time to disease decreased from  $481 \pm 4$  days in the 2<sup>nd</sup> passage to  $318 \pm 10$  days in the 3<sup>d</sup> (Fig. 1A). The 4<sup>th</sup> passage incubation time was not statistically different from that of the 3<sup>d</sup> passage, suggesting that it is stabilized by the 3<sup>d</sup> passage (Fig. 1A). This incubation time was different from those of currently known hamster-adapted TSE strains.

In previous studies, very slow disease progression after the first clinical signs was observed for the SSLOW 2<sup>nd</sup> passage (25). In a similar manner, the animals of the 3<sup>d</sup> and 4<sup>th</sup> passages also showed very slow progression of symptoms (Fig. 1A). The clinical stage lasted for approximately 90 to 150 days after disease onset before reaching a terminal stage. The first indication of clinical onset was a non-habituating startle response to sound and touch and an agitated, fidgeting behavior. As disease progressed, animals were unable to rear unassisted by a cage wall and had increasing difficulty righting themselves when rolled onto their back. During the progression, the hair became very dry standing out from the body and could be easily detached in large clumps even with gentle handling. By the terminal stage, the animals had become less active in general, and when active their movements were much slower. Overall, serial transmission of SSLOW revealed that strain-specific clinical features were preserved and that long incubation time to onset and very slow disease progression represents the clinical hallmark of this strain.

As reported previously (25), obesity was one of the most notable clinical features of SSLOW-infected animals. Consistent with previous observations, the majority of animals from the 3<sup>d</sup> passage weighed significantly more than that of a control group by 300 days postinoculation (Fig 1B). Although other clinical signs were not yet detectable at this time point, weight gain alone might not serve as a reliable early marker of the clinical disease, because a small fraction of SSLOW-inoculated animals have never gained a weight higher than that of a control group. Nevertheless, regardless of whether SSLOW-inoculated animals were overweight or not, they showed substantial weight fluctuation during clinical disease, while the weight of individual animals within age-matched control group remained stable.

*Histopathological study revealed gradual stabilization of SSLOW-specific features* - As judged from the lesion profile and PrP immunoreactivity score, animals from the 2<sup>nd</sup>, 3<sup>d</sup> and 4<sup>th</sup> passages exhibited similar neuropathological features (Fig. 2 A,B). These features, however, continued to evolve for as far as the 4<sup>th</sup> passage. In SSLOW-inoculated animals from the 2<sup>nd</sup>, 3<sup>d</sup> and 4<sup>th</sup> passages, the spongiform changes, neuronal loss, and reactive astrogliosis were prominent in the thalamus, caudate-putamen, brainstem, and deeper layers of the cerebral cortex, but relatively mild in the hippocampus and cerebellum (Fig. 2 A). However, the 4<sup>th</sup> passage animals exhibited more substantial lesions in the cerebellar granular layer than animals of the 2<sup>nd</sup> and 3<sup>d</sup> passages. (Fig. 2 A). The region-specific PrP deposition profile was similar in animals from all three passages and consisted of synaptic and perineuronal deposits, as well as large plaques of 25-60  $\mu$ m size in the subependymal regions (Fig. 2 B, I-N). Again, 4<sup>th</sup> passage animals exhibited substantially more prominent PrP deposition in most areas including the cerebellum, thalamus, and hippocampus than those of the 2<sup>nd</sup> or 3<sup>d</sup> passages (Fig. 2B-K). Moreover, while animals from the 2<sup>nd</sup> and 3<sup>d</sup> passages showed significant variations in the intensity of deposition within the same animal group, the animals from the 4<sup>th</sup> passage showed very uniform PrP immunoreactivity between animals (Fig. 2 B).

Previously we did not observe any signs of PrP deposition in spleens in the animals of the 2<sup>nd</sup> passage that led us to conclude that SSLOW is not lymphotropic. Surprisingly, small amounts of PrP immunodeposition were found in the spleens of 4<sup>th</sup> passage animals (Fig. 2 O,P). These experiments demonstrated that SSLOW histopathological features including a tropism to lymphoid tissues continue to evolve during serial transmission even after the incubation time to disease has stabilized.

*SSLOW-specific dose dependence* - To establish a SSLOW-specific dose-response curve, two ten-fold serial dilutions were prepared using BHs of two terminally sick animals from the 2<sup>nd</sup> passage and each dilution was inoculated into a group of four animals. For the animals inoculated with high doses (10- to 10<sup>4</sup>-fold diluted BH), the incubation period to the initial clinical signs showed a very modest increase with serial dilution (Fig. 3A,B). However, the progression to a terminal

stage after the first signs was much slower for the animals inoculated with low doses (Fig. 3B). The animals inoculated with  $10^5$ -fold and higher dilutions showed a much stronger dose dependence of incubation time to disease (Fig. 3). An incomplete attack rate was observed for  $10^6$ - and  $10^7$ -fold dilutions, while no animals inoculated with  $10^8$ -fold or higher dilutions showed clinical signs (Table 1). A point of inflection, which is normally observed in the end-point titration experiments (31), occurred between  $10^4$  and  $10^5$ -fold dilutions.

To test whether any asymptomatic animals inoculated with low doses were infected, Western blot and serial PMCAb (sPMCAb) were employed to detect PrP<sup>Sc</sup> in their brains. Six PrP<sup>Sc</sup>-positive brains were identified by Western blot in a group of asymptomatic animals that were inoculated with  $10^6$ -,  $10^7$ - or  $10^8$ -fold diluted brain material (Fig. 4, Table 1). On the other hand, one animal (# 366) that was clinically ill showed a barely visible, if any at all, signal on Western blot (Fig. 4 and 6). Furthermore, brains from an additional seven asymptomatic animals that were negative on Western blot were found to contain small amounts of PrP<sup>Sc</sup> in experiments where their brain material was used to seed PrP<sup>Sc</sup> amplification in sPMCAb consisted of three rounds (Fig. 5, Table 1). sPMCAb seeded with BHs from age-matched controls were used in each sPMCAb experiments and were always negative (data not shown). These results illustrate that (i) inoculations with low doses could lead to subclinical prion infection and (ii) asymptomatic animals might harbor various amounts of PrP<sup>Sc</sup>, from as large as typically found in the terminally ill animals to as little as those that could only be detected by sPMCAb.

*Dissociation between clinical disease and amounts of PrP<sup>Sc</sup> in animals inoculated with low-dose inocula* - At the high dose inocula (from  $10^1$  to  $10^5$ -fold diluted BHs), all animals developed clinical disease and showed large amounts of PrP<sup>Sc</sup> in their brains. The result illustrates a nice correlation between accumulation of PrP<sup>Sc</sup> and occurrence of clinical signs. In contrast, in animals inoculated with the low doses (from  $10^6$ - to  $10^8$ -fold diluted BH), a dissociation between PrP<sup>Sc</sup> amounts in brains and clinical status was observed. On one hand, several animals (# 313, 314, 317, 357 and 359) harbored PrP<sup>Sc</sup> in amounts comparable to those seen in brains of terminally ill animals, yet remained asymptomatic (Fig. 4). On the other hand,

one animal (# 366) was found to be clinically ill, but had very little if any PrP<sup>Sc</sup>, as judged by Western blot (Fig. 4, 6). Together, these results revealed a lack of correlation between PrP<sup>Sc</sup> amounts and the clinical status in animals inoculated with the low doses.

To test whether PrP<sup>Sc</sup> from the animal #366 was intrinsically more sensitive to PK digestion than PrP<sup>Sc</sup> from other brains, BHs were treated with a broad range of PK concentrations. Surprisingly, at low PK concentrations the PK-resistant signal did not increase (Fig. 6). This result supports the notion that the animal #366 harbored very small amounts of PK-resistant PrP<sup>Sc</sup> rather than PK-sensitive PrP<sup>Sc</sup>.

To test whether clinical status correlates with the number of PMCAb-reactive PrP<sup>Sc</sup> particles, BHs from the animal #365 (symptomatic, large amounts of PrP<sup>Sc</sup>), #366 (symptomatic, miniscule amounts of PrP<sup>Sc</sup>) and #317 (asymptomatic, large amounts of PrP<sup>Sc</sup>) were subjected to PMCAb titration as previously described (29). BHs were serially diluted 10-fold up to  $10^{13}$ -fold, and each dilution was used to seed sPMCAb. In sPMCAb consisting of three serial rounds, the reactions seeded with as high as  $10^{10}$ -fold and  $10^9$ -fold diluted BHs #365 and #317, respectively, were positive (Fig. 7A). Surprisingly, sPMCAb reactions seeded with  $10^4$ - to  $10^{13}$ -fold diluted brain material from animal #366 were all negative (Fig. 7A). To test whether the brain #366 contained any PMCAb reactive material at all, the reactions were seeded with a series of low dilutions. No amplification was detected in reactions seeded with brain material #366 diluted  $10^3$ -fold or higher in sPMCAb consisting of three rounds (Fig 7B). In comparison, the reactions seeded with  $10^4$ - and  $10^5$ -fold diluted brain materials from animal #365 or #317 were positive after the first and second rounds, respectively. These experiments showed that brain #366 had very small amounts of PMCAb reactive material and/or that, in contrast to PrP<sup>Sc</sup> from the rest of SSLOW-inoculated animals, PrP<sup>Sc</sup> from #366 is not readily amplifiable by PMCAb.

## DISCUSSION

In previous studies we reported that transmissible prion disease could be generated in Syrian hamsters upon inoculation of amyloid fibrils prepared *in vitro* from full-length recombinant PrP (25-27). While rPrP fibrils displayed very limited infectivity, the prion disease that emerged in the

second or third passages showed very unique phenotypes. This was significantly different from other approaches for generating prion disease *de novo* that involved conversion of PrP<sup>C</sup> or rPrP into PrP<sup>Sc</sup> in non-seeded PMCA supplemented with brain homogenate or cellular cofactors (3,4,19). Because PrP<sup>Sc</sup> generated in PMCA reactions with hamster PrP<sup>C</sup> produced disease phenotypes similar to those of two of the most commonly employed hamster-adapted prion strains, 263K and Hyper (19,20), it was proposed that hamster PrP displays a natural tendency to adopt 263K- or Hyper-specific PrP<sup>Sc</sup> folding pattern (20). The question of great interest is whether the unique disease phenotype that emerged upon fibril inoculation in our studies is sustainable in a serial transmission or transforms into a phenotype specific for one of the existing hamster-adapted strains. If the latter is true, the unique features observed for SSLOW 2<sup>nd</sup> passage would have to be interpreted as a stage in the evolution of a more stable strain-specific phenotype.

To address this question, serial transmission of SSLOW was performed. The incubation time reduced from 485±5 days at the 2<sup>nd</sup> passage to 318±16 days at the 3<sup>d</sup> passage but did not show any further drop at the 4<sup>th</sup> passage. This result argues that the incubation time to disease is stabilized by the 3<sup>d</sup> passage and that SSLOW is characterized by an intrinsically long incubation time which is different from those of known hamster-adapted strains (5). Furthermore, regardless of a serial passage number SSLOW-infected animals consistently showed very slow progression of clinical disease. While the duration of a clinical stage varied in individual animals from ~90 to 150 days, the mean duration remained stable during serial passages (Fig. 1A). An alternative explanation for a slow disease progression is that in SSLOW-inoculated animals the first noticeable clinical signs were observed at an earlier disease stage than in animals inoculated with experimental rodent-adapted strains. Nevertheless, the long incubation time to the terminal stage supports the previous conclusion that rPrP fibrils produce unique disease phenotypes.

A number of pathogenic features including long incubation time to and duration of clinical disease and weight gain were found to be peculiar for SSLOW. Obesity was previously reported in animals inoculated with 139H hamster-adapted

scrapie agent (32). However, unlike 139H, in addition to overall weight gain SSLOW-inoculated animals exhibited significant individual weight fluctuation during the clinical stage (Fig. 1B). Moreover, a fraction of SSLOW-inoculated animals never gained weight above the control group or showed substantial weight loss after weight gain. These results suggest that the mechanism behind prion-induced impairment in energy homeostasis in SSLOW and 139H is different. Furthermore, SSLOW- and 139H-specific PrP<sup>Sc</sup> display dramatically different amplification efficiencies in PMCA. 139H PrP<sup>Sc</sup> is characterized by a very poor amplification rate (5,33), whereas the amplification rate of SSLOW PrP<sup>Sc</sup> was found to be almost as high as that of 263K PrP<sup>Sc</sup> (30,33). In summary, the current studies demonstrated that rPrP fibrils produced a unique disease phenotype, and that its unique features were preserved during serial transmission.

Surprisingly, while the incubation time was found to stabilize at the 3<sup>d</sup> passage, the histopathological assays revealed a notable increase in intensity of PrP deposition in most brain areas in animals of the 4<sup>th</sup> passage comparing to those of the 3<sup>d</sup> passage. Furthermore, PrP immunoreactivity was observed in spleens of animals from the 4<sup>th</sup> passage. While all currently known hamster-adapted strains target lymphoid tissues, PrP immunoreactivity was not detected in animals from the SSLOW 2<sup>nd</sup> passage (25). These results suggest that PrP<sup>Sc</sup> properties continue to evolve for as far as four serial passages and that PrP<sup>Sc</sup> invasion of brain regions and peripheral tissues become more rapid and/or more aggressive with serial transmission. Previously, upon transmission and adaptation of prions to a new host, lymphoid tissues were found to be more permissive than brain to prion replication (34). Opposite to the high susceptibility of lymphoid tissues to prion invasion upon cross-species transmission, the current study shows that lymphotropism emerged much later when a new strain evolved within the same host. It is possible, however, that the initial lack of lymphotropism was a reflection of an inoculation route. Because rPrP fibrils were first exposed to brain tissues, the newly emerged PrP<sup>Sc</sup> population was set to adapt to a brain environment. We do not know whether the initial tissue tropism would be different if peripheral inoculation routes were used.



Gradual change in strain properties including neurotropism are frequently observed upon cross-species prion transmission in a phenomenon referred to as strain adaptation (6,7,15,35). The process of strain adaptation to a new host can occur for several serial passages and is attributed in large part to adaptation of the strain-specific structure of donor PrP<sup>Sc</sup> to the amino acid sequence of the acceptor PrP<sup>C</sup>. In a manner similar to strain adaptation, several serial passages were required to stabilize SSLOW-specific features in the current study. However, in contrast to an adaptation that accompanies cross-species transmission, the process of SSLOW ‘adaptation’ was observed in the absence of changing the host species.

The current study illustrates that the phenomenon of prion strain adaptation is more common than has been generally thought. It could be observed upon serial prion transmission within the same host and in the absence of changes in the PrP amino acid sequence or prion replication environment. Presumably, the adaptation within the same host was attributed to the fact that rPrP amyloid fibrils that give rise to synthetic strains display a folding pattern substantially different from those observed in PrP<sup>Sc</sup>. Because replication of rPrP amyloid structure is cofactor independent, whereas replication of PrP<sup>Sc</sup> is co-factor dependent (33,36,37), adaptation could also be attributable to transformation of cofactor independent to cofactor dependent self-replicating PrP structures. The last hypothesis seems particularly attractive considering recent data that cellular cofactors including lipids might play an active role in defining strain-specific features (38,39). The adaptation of synthetic strains within the same host was likely to involve a selection and evolution of a PrP<sup>Sc</sup> subpopulation that was best suited to replicate in a particular cellular environment, such as the brain or lymphoid tissues.

In transmissible prion diseases, the incubation time to disease correlates with the inoculated dose in a predictable manner (40,41). In the current studies, animals inoculated with high doses of SSLOW showed a very modest dose-dependence for the incubation time to disease (Fig. 3B). In fact, the incubation time was not statistically different between the four groups that received 10<sup>-</sup>, 10<sup>2</sup>-, 10<sup>3</sup>- or 10<sup>4</sup>-fold diluted SSLOW BHs (Fig. 3A,B). However, the mean duration of

clinical stage increased significantly with serial dilution of inocula (Fig. 3B). If a total time to terminal stage is used instead of the incubation time to initial clinical signs, SSLOW titration followed a dose-response relationship typically observed in titration experiments. Noteworthy, an increase in duration of clinical stage as a function of inoculum dose was previously reported for other rodent strains including RML (41). We do not know whether the long time between first signs and terminal stage in SSLOW-inoculated animals could be attributed to the intrinsically slow progression of the clinical stage or to the possibility that the first noticeable signs of the disease are detectable at much earlier stages than in animals infected with other TSE strains.

If clinical signs were used as a sole read-out parameter for estimating infectivity titer, 10<sup>6</sup>-fold diluted SSLOW brain material corresponded to a limiting dilution at which only 50% of animals developed the disease (Table 1). In agreement with the dose-response curves reported for other TSE strains (31), an inflection point occurred at approximately 10<sup>2</sup> units of infectivity above the limiting dilution established by clinical criteria. The physical basis for the inflection point in a dose-response curve remains unclear. Upon intracerebral inoculation, only a small fraction of infectious material appears to settle down in the brain, while a significant part of it either cleared or moved out from the brain into peripheral organs and tissues (42). One can speculate that at low inoculum doses, the brain is not infected immediately, but rather after prions leave the brain, infect peripheral organs, and then re-invade the central nervous system. If this mechanism is correct, it explains the prolonged incubation time and stochastic scattering of individual incubation times observed at low doses. As an indirect support of this mechanism, recent studies revealed that following cross-species intracerebral inoculations, prions were found to replicate more effectively in lymphoid tissues than in brain (34).

In agreement with previous studies (29,41,43), the current work revealed that the infectivity titer is likely to be underestimated if appearance of clinical signs is used as sole read-out parameter. Indeed, upon application of Western blot and sPMCAb, substantial fractions of animals inoculated with 10<sup>6</sup>- to 10<sup>8</sup>-fold diluted brain material were found to be infected without

displaying any clinical signs (Table 1). In fact, sPMCAb improved prion detection limits in animals inoculated with low doses by approximately  $10^2$ -fold beyond the limiting dilution established by occurrence of clinical signs. Detection of PrP<sup>Sc</sup> by PMCAb revealed that animals inoculated with small doses represent asymptomatic prion carriers for the animals' lifetime. Notably, this result resembled persistent prion infections observed in the absence of clinical signs upon cross-species transmission (44-46). However, in contrast to the low inocula doses that gave rise to subclinical SSLOW infection, high dose inocula were used in inter-species transmission experiments. Considering that the half life of PrP<sup>Sc</sup> is relatively short (42,47), it is unlikely that SSLOW PrP<sup>Sc</sup> detected by sPMCAb at more than 600 days postinoculation represents the original material inoculated at high dilutions. Notably, the asymptomatic animals showed significant variations in the amounts of PrP<sup>Sc</sup>. Taken together, these results highlight the stochastic nature of events leading to PrP<sup>Sc</sup> replication and accumulation at low inocula doses.

100% of animals inoculated with high doses ( $10^1$ - to  $10^5$ -fold diluted BHs) were found to harbor large amounts of PrP<sup>Sc</sup> and develop prion disease. However, at the low doses ( $10^6$ - to  $10^8$ -fold diluted BHs), large amounts of PrP<sup>Sc</sup> were observed in the absence of clinical signs in several animals, whereas one symptomatic animal had very little, if any PrP<sup>Sc</sup>. It is likely that animals inoculated with the low doses require longer time to reach a toxic threshold to trigger clinical stage. Therefore, these results might suggest that old animals are less susceptible to PrP<sup>Sc</sup> toxicity or need a higher PrP<sup>Sc</sup> threshold. These data might also suggest that only a small sub-fraction of PrP<sup>Sc</sup> is neurotoxic. In SSLOW-infected animals, a substantial fraction of PrP<sup>Sc</sup> is deposited in the form of large plaques suggesting that large deposits are less toxic to neurons than small, diffuse deposits. Nevertheless, the current work supports the hypothesis that two distinct processes, one being responsible for PrP<sup>Sc</sup> replication and another for accumulation of toxic species, exist (28). While accumulation of toxic PrP species was tightly coupled to PrP<sup>Sc</sup> replication in animals inoculated with high doses, these two processes appear to be in disarray at the low dose inocula. It is unclear whether toxic PrP species are intermediate or byproducts of PrP<sup>Sc</sup> replication, or

whether they are catalyzed by or formed independently of PrP<sup>Sc</sup>.

One of the most intriguing results of the current study was an observation of a very small PrP<sup>Sc</sup> amount in the clinically ill animal #366. Even a substantial decrease in PK concentration did not lead to a higher PrP<sup>Sc</sup> signal on Western blot for this animal. Furthermore, a series of PMCAb experiments revealed that the brain #366 contained very low titers of PMCAb reactive material and/or that the PrP<sup>Sc</sup> harbored in the brain #366 was not readily amplified in PMCAb, unlike PrP<sup>Sc</sup> from other SSLOW-inoculated animals. On the other hand, a high titer of PMCAb-reactive material was found in an asymptomatic brain (animal #317). Again, these results support the hypothesis that the two processes, one leading to accumulation of PMCAb-reactive material or PrP<sup>Sc</sup> and another to accumulation of toxic PrP species are distinct and dissociated. An alternative possibility to consider for explaining the puzzle with the animal #366 is that serial passage of SSLOW at a low dose resulted in an isolation of a new strain. In previous studies, serial passages of TSE agents at high and low doses often produced two different strains, a phenomenon referred to as strain breakdown or mutation (9,13,15,16,18). Typically, strain breakdown was observed upon cross-species transmission and adaptation to a new host (9,13,15,18). Nevertheless, consistent with the hypothesis that prion strains consist of quasi-species (48,49), prion replication in a new cellular environment might lead to isolation of a new strain that was present in small amounts in the original inoculums. One could speculate that inoculation of rPrP fibrils resulted in a mixture of PrP<sup>Sc</sup> conformations in animal brain, from which distinct strains are selected depending on inocula dose in the course of serial passages. Changes in the physical properties of PrP<sup>Sc</sup> populations during serial transmission are consistent with this hypothesis (23,25,50). Nevertheless, it remains to be determined in future studies whether the animal #366 harbors a strain different from SSLOW.

While infectivity of rPrP fibrils generated *in vitro* was very low, peculiar conformation of rPrP fibrils gave rise to a panel of new prion strains including SSLOW and LOTSS with unique disease phenotypes (25-27). These new strains of synthetic origin represent valuable new models of TSE

disease, which are worth of exploiting for addressing a number of questions in prion biology.

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**Table 1. Comparison of the titration results for low dose inocula**

Inoculum dilution	Clinical TSE / total inoculated	Positive in Western blot / total inoculated	Positive in sPMCAb <sup>a</sup> / total inoculated
10 <sup>-5</sup>	5/5 <sup>b</sup>	5/5	5/5
10 <sup>-6</sup>	4/8	5/8	7/8 <sup>c</sup>
10 <sup>-7</sup>	1/8	4/8	8/8
10 <sup>-8</sup>	0/8	1/8	2/8

<sup>a</sup>The results are based on appearance of PK-resistant positive signal after three sPMCAb rounds

<sup>b</sup> Three animals from this group succumbed to unrelated illness.

<sup>c</sup> One out of seven BHs with positive PMCAb seeding activity showed very low amplification rate.

## FIGURE LEGENDS

**Figure 1. Serial transmission of SSLOW.** (A) Mean incubation time to disease onset (light gray bars) and mean duration of clinical stage after the appearance of first clinical signs (dark gray bars) as a function of SSLOW serial passage. (B) Change in body weight during the clinical stage in individual animals inoculated with 10% SSLOW BH (thin red lines) or with 10<sup>11</sup>-fold diluted SSLOW BH used as a control group (thick black lines). No animals inoculated with 10<sup>11</sup>-fold diluted BH showed clinical signs or PrP<sup>Sc</sup> in their brain material. The results are from the 3<sup>d</sup> SSLOW passage.

**Figure 2. Histopathological analysis.** (A) Lesion profile obtained by averaging scores of spongiform change, neuronal loss and gliosis, and (B) PrP immunopositivity score for each anatomical region in hamsters of the 2<sup>nd</sup>, 3<sup>d</sup>, and 4<sup>th</sup> SSLOW passages. The lesion profile was obtained by averaging the scores for spongiform change, neuronal loss, and gliosis for three animals within each group. Overview (x250) of the hippocampus (C-E), thalamus (F-H), cerebellum (I-K), and subependymal region (L-N) using immunostaining for disease-associated PrP in animals from the 2<sup>nd</sup> (C, F, I, L), 3<sup>d</sup> (D, G, J, M), or 4<sup>th</sup> (E, H, K, N) SSLOW passages. High magnification (x400) images of PrP immunostaining in the spleen (O, P) in animals from the 2<sup>nd</sup> (O) and 4<sup>th</sup> (P) SSLOW passages. Scale bar 15 μm in C-E and I-K, 25 μm in F-H and L-N, and 10 μm in O, P.

**Figure 3. Relationship between inoculation dose and incubation time.** (A) Incubation time to disease as a function of inoculum dose for two independent serial dilutions (shown as triangles and circles) prepared from brain material from two animals from 2<sup>nd</sup> passage of SSLOW. Each serial dilution was inoculated into a group of four animals for each titration experiment. Dashed line indicates an inflection point. (B) Mean incubation time to disease onset (light gray bars) and mean duration of the clinical stage of the disease after the first symptoms (dark gray bars) as a function of inoculum dose for two independent serial dilutions. Three animals in these group marked by asterisk succumbed to illnesses not related to TSE.

**Figure 4. Western blot analysis of PrP<sup>Sc</sup> in animals inoculated with low doses.** Western blot of BHs from animals of SSLOW 3<sup>d</sup> passage inoculated with 10<sup>-4</sup> to 10<sup>-8</sup>-fold diluted BHs as indicated. N marks animals that did not show any clinical signs, and C marks animals that progressed to clinical stage. Asymptomatic animals were euthanized at 685 dpi. Samples were treated with 20 μg/ml PK, 3F4 antibody was used for staining.

**Figure 5. Detection of PrP<sup>Sc</sup> in animals inoculated with low doses using PMCAb.** Western blots of products of sPMCAb reactions seeded with BHs from the animals of SSLOW 3<sup>d</sup> passage inoculated with 10<sup>-6</sup>-, 10<sup>-7</sup>- or 10<sup>-8</sup>-fold diluted BHs as indicated. sPMCAb consisted of three rounds, 3F4 antibody was used for staining.

**Figure 6. Analysis of PrP<sup>Sc</sup> resistance to PK.** BHs from animals inoculated with 10<sup>-6</sup>-fold diluted BHs were treated with increasing concentration of glycerol-free PK as indicated for 1

hour at 37°C. **N** marks animals that did not show any clinical signs, and **C** marks animals that progressed to clinical stage. Western blots were stained with 3F4.

**Figure 7. Analysis of PMCAb-reactive material. (A)** PMCAb titration of BHs from animals inoculated with  $10^6$ -fold diluted BHs. BHs from animals # 365, 366 or 317 were serially diluted up to  $10^{13}$ -fold into 10% NBH as indicated and subjected to four sPMCAb rounds. Nine non-seeded control sPMCAb reactions are shown. **(B)** BHs from animals # 365, 366 or 317 were serially diluted up to  $10^5$ -fold into 10% NBH as indicated and subjected to one, two or three sPMCAb rounds. Undigested 10% NBH is provided as a reference (-PK). Western blots were stained with 3F4.

Figure 1

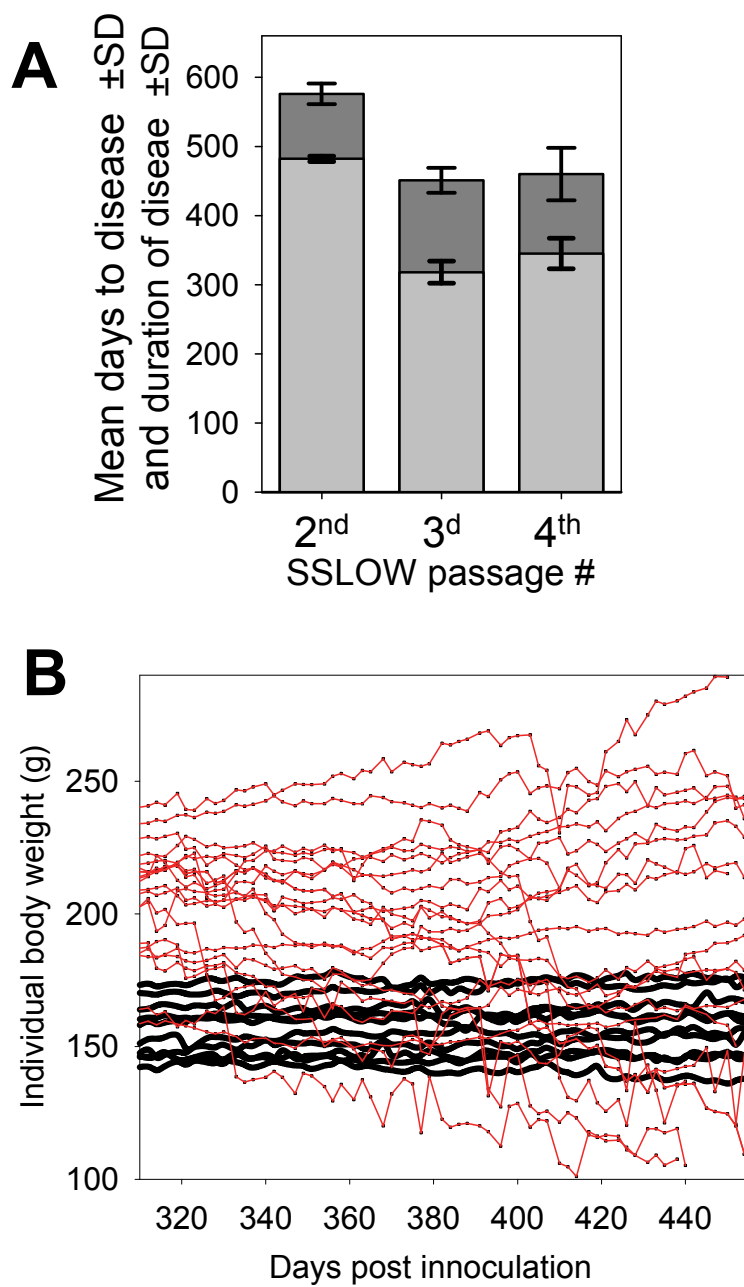




Figure 2

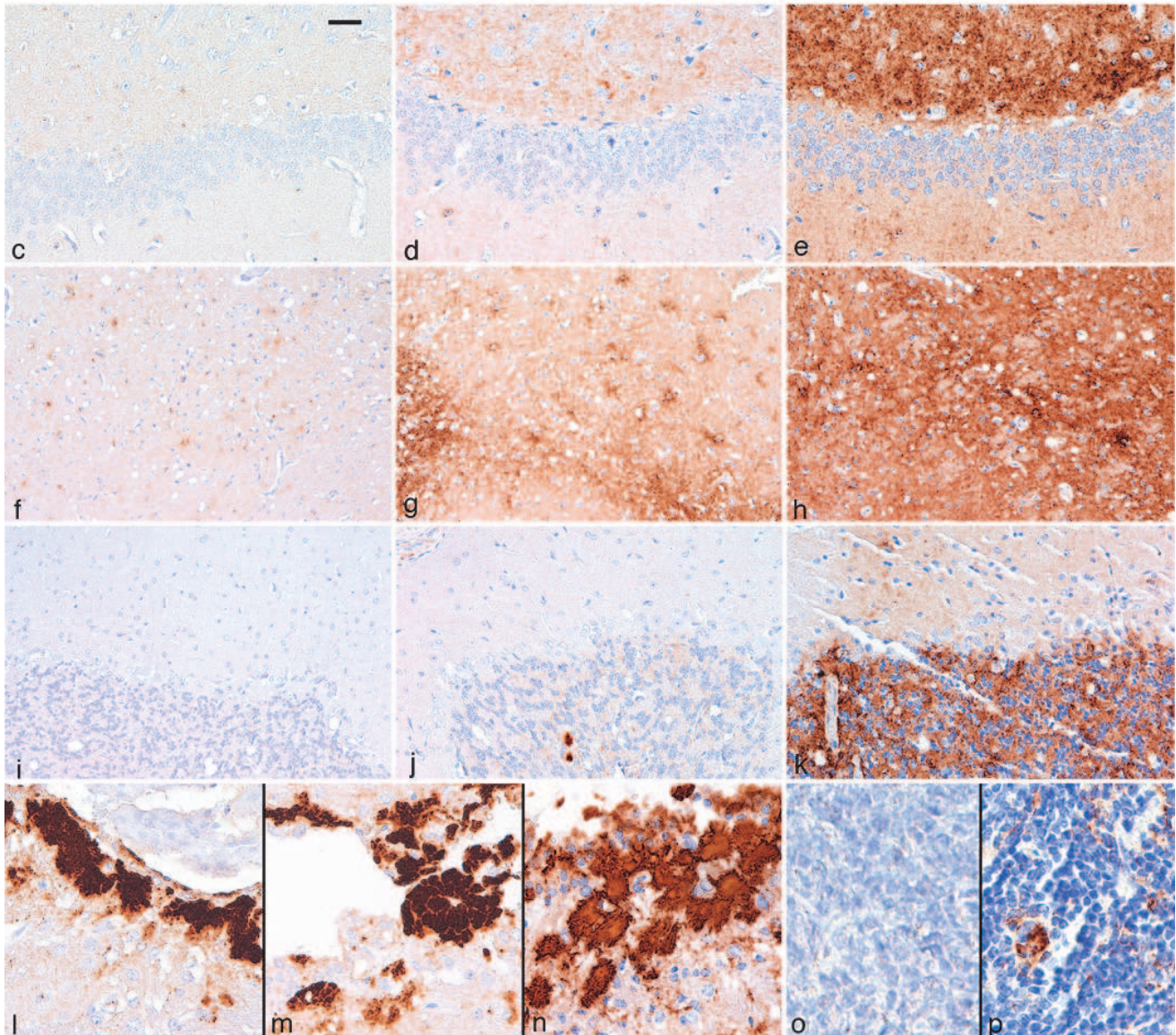
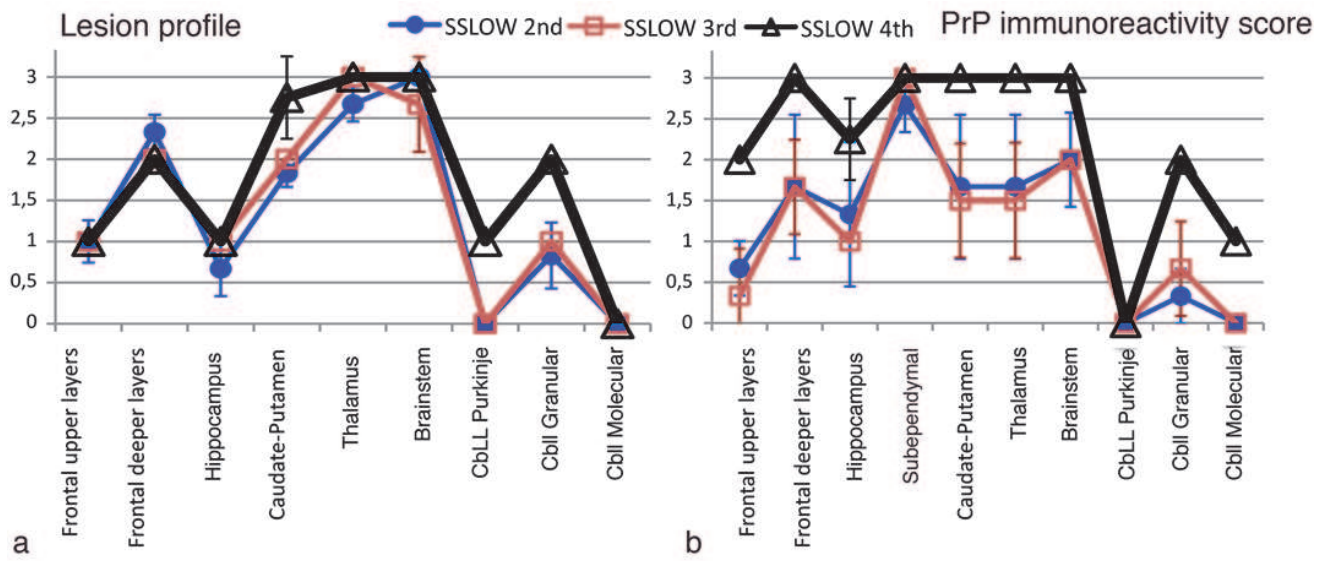




Figure 3

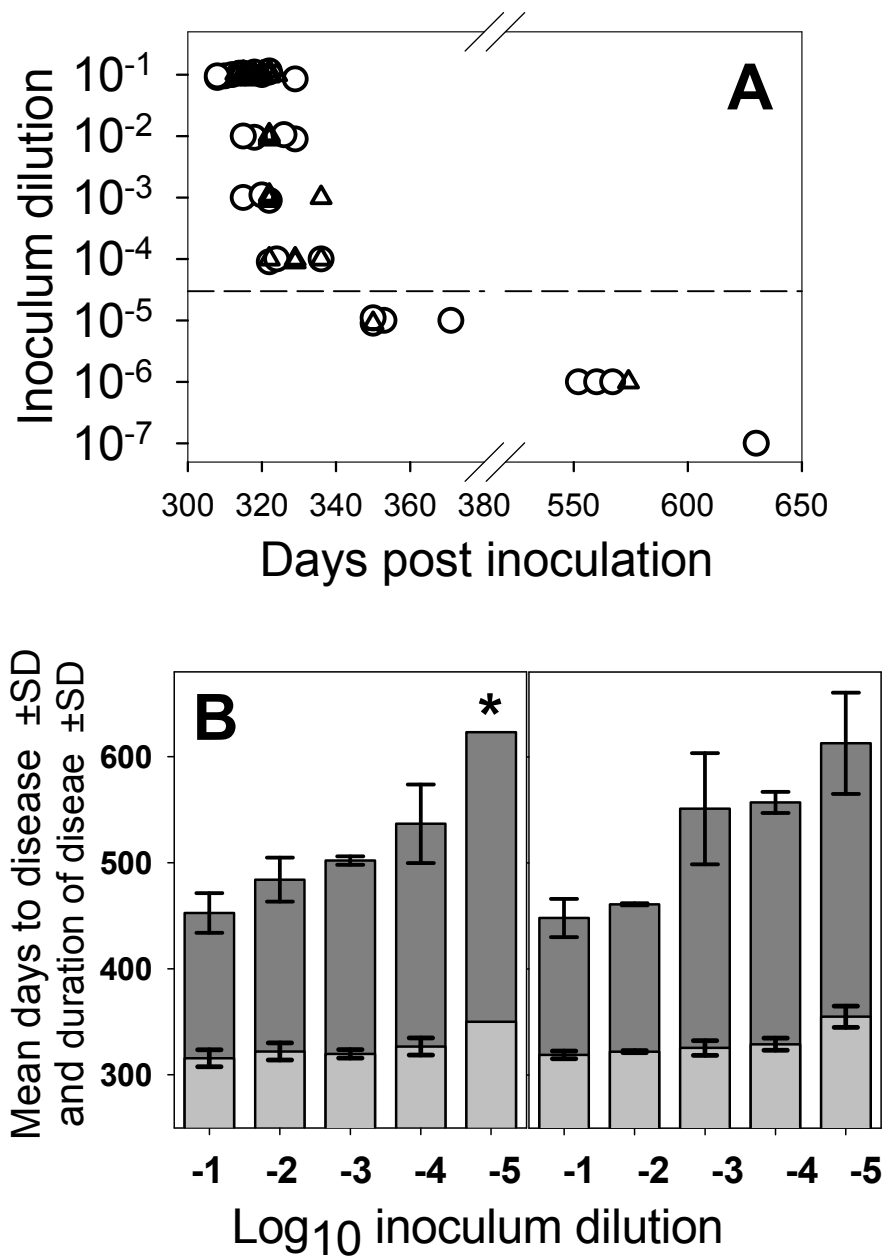


Figure 4

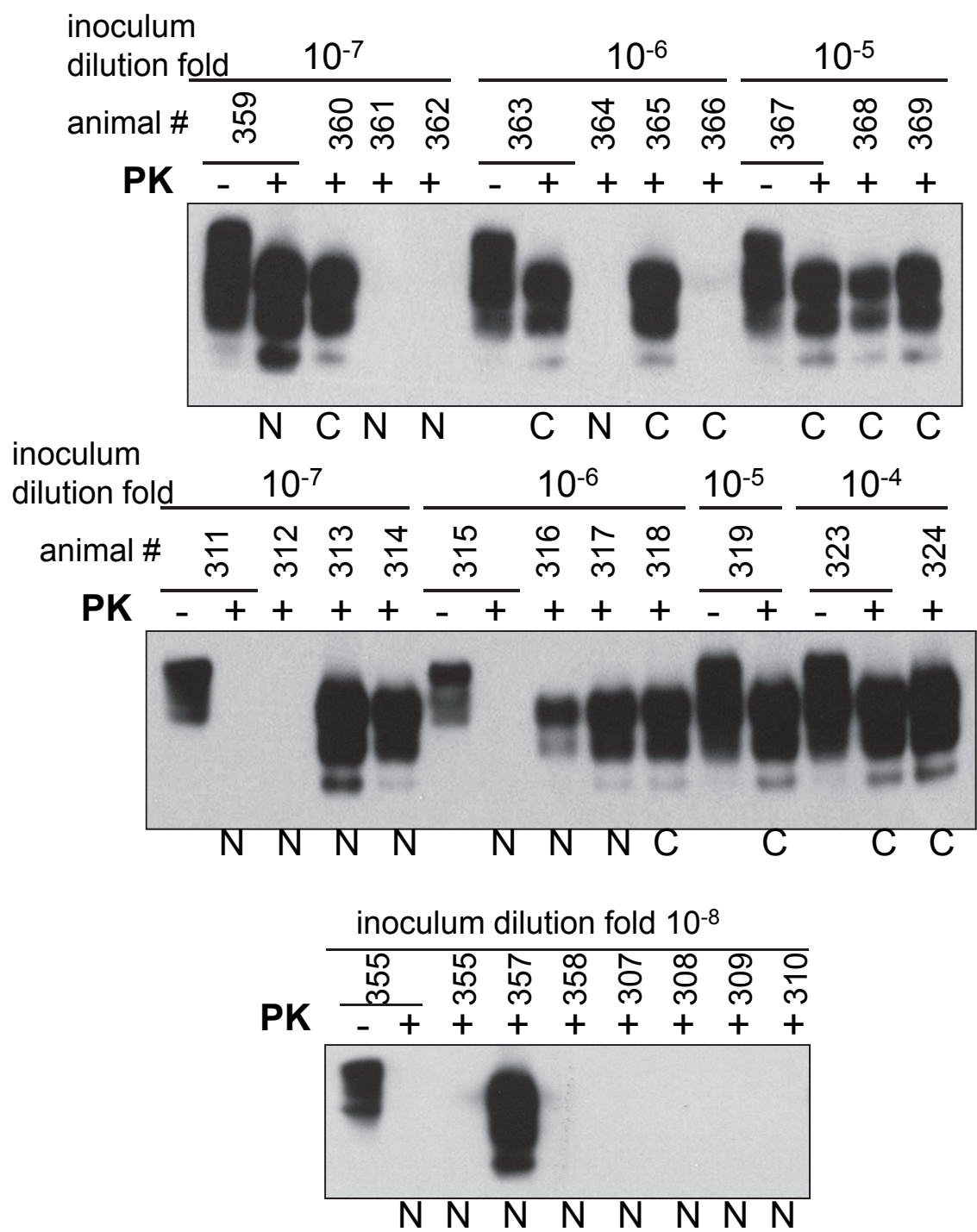


Figure 5

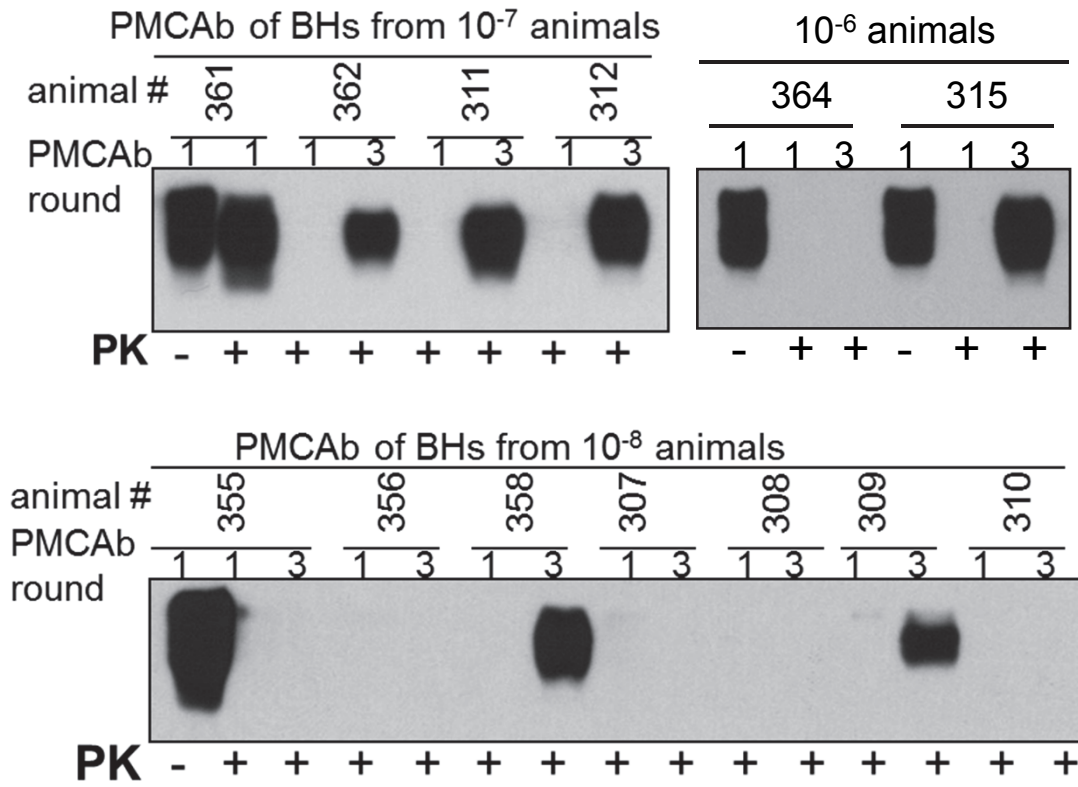


Figure 6

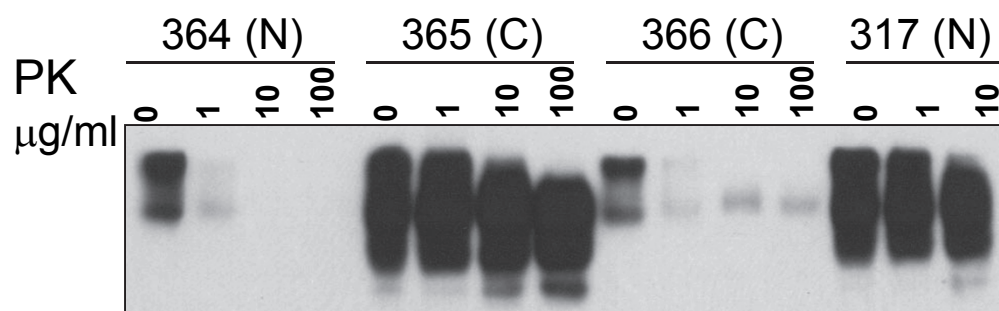




Figure 7

